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14. ABSTRACT This study has rested on the basic hypothesis that agents given during and shortly after resuscitation can favorably influence the systemic inflammatory response to hemorrhagic shock and injury. This hypothesis has been shown to be true in the animal model. Of possible therapeutic agents possible, three were identified for potential human use. Glutamine was the first to be used in patients. Clinical studies have not supported the use of this agent. Arginine has been successful in the animal model, but has not been used in for this purpose in humans. Clinical studies in the literature are mixed regarding its safety in shock. Allopurinol has been shown in the past to be successful, in animal models, but recent experiments have failed to confirm benefit. Studies were also carried out using alanine-glutamine dipeptide, which has substantial advantages over glutamine. But clinical studies by others have failed to show benefit of this agent. It is used widely in Europe, but the US FDA has not approved it for use, and it is unlikely to become available for US use.						
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INTRODUCTION

Shock is a leading cause of death among American soldiers wounded in battle. Unless injuries are immediately lethal, most deaths result from hemorrhagic shock or from its late sequelae, septic shock and multiple organ failure. The critical time in shock appears to be during and shortly after resuscitation. Resuscitation is associated with a generalized activation of the systemic inflammatory reaction. This has a number of adverse effects, including immunosuppression, and renders the patient vulnerable to sepsis and its sequelae. The *overall goal* of this research program is to develop new treatments for hemorrhagic shock to be administered shortly after injury, during or around the time of initial resuscitation, to suppress or at least modulate this systemic inflammatory reaction. If the program is successful, treatments will be developed that can be applied both by front-line responders on the battlefield and by fixed facilities such as Forward Surgical Teams or Combat support Hospitals. Equally, these will be available to first responders and trauma centers in civilian life,

The choice of suitable agents is limited by a number of factors. Most importantly, agents must be non-toxic and have a very broad therapeutic ratio, so that they can be given safely to injured soldiers. They must be easy to administer under combat conditions. In previous work, a number of agents have been studied. Allopurinol, a xanthine oxidase inhibitor, has been found beneficial in an experimental model of shock, as long ago as the 1980's (1). In other experimental work (2), glutamine, glutamine-alanine dipeptide, and arginine have shown efficacy. (3, 4) Studies with crocetin have also shown efficacy (5). Unfortunately, this last agent is not available in a form that can be administered intravenously. Others have advocated the use of DHEA, but studies in our laboratories have failed to show a useful effect (6, 7). Studies with omega-3 fatty acids have shown promise, but are still at an early stage (8). In contrast to intravenous use, the practicality of which varies for these agents, all can be given orally. This raises the possibility that they could be given prophylactically to personnel who are at risk for injury. Finally, administration of glucose and insulin has shown considerable benefit in the experimental model (9). While such administration may be too complex for field administration, it may prove to be a useful adjunct to resuscitation in fixed facilities.

The *primary method* of this research has been to use these drugs in the rodent shock model, as in most of the findings reported above. A crucial outcome measure has also been the effect of shock, resuscitation, and supplemental treatment upon adenosine nucleotides (1, 3, 5, 6). More recently, research has been carried out to determine the response of the genome to hemorrhagic shock, using both whole-genome microarray studies and studies of individual genes using the real-time polymerase chain reaction technique (RT-PCR). A *secondary method* of this research has been developed as a cell-culture model of hypoxia. Many of the deleterious effects of shock are caused by lack of perfusion of cells and organs. To simulate this low-perfusion state in a cellular model can be done by subjecting the cells to lower than normal levels of oxygen, and observing the effect on such things as cellular ATP and cytokine production. While this work has been funded by local resources, some of the findings which have been generated are strongly applicable to the present project.

Clinical research was initiated in cooperation with the Truman Medical Center Trauma Service. While these studies have entered only a few patients, the major purpose of this patient research was to assess whether glutamine will be effective in treating the patient who is post-resuscitation from shock and injury.

BODY

In the Shock Trauma Research Center, we are in the process of developing therapeutic strategies based either on known pharmacologic agents (allopurinol, crocetin) or on nutrients with pharmacologic properties (arginine, glutamine, alanine-glutamine dipeptide, omega-3 fatty acids, glucose/insulin). The basic aim has been to reduce the intensity of the post-shock and post-injury inflammatory response. As noted above, we have focused on treatment strategies involving one or more agent(s) that can be given early in resuscitation, with complete safety, even by first responders and before formal resuscitation and treatment has begun. Of importance, these restrictions means that the choice of possible agents is limited

The identification of such agents, which can be given at the beginning of resuscitation, or even before fluid resuscitation is begun, has been the subject of a great deal of research. In our own laboratories, during previous studies using several experimental animal models of hemorrhagic shock, we have identified several compounds that have shown therapeutic effectiveness, as outlined in the introduction. The most promising of these are arginine, glutamine, and arginine dipeptide. As outlined in the previous paragraph and the Introduction, we have identified a number of other agents (1-10). In the case of each agent, there is convincing experimental evidence that it enhances cellular recovery following shock, and, importantly, that it significantly improves survival in the experimental animal model. These agents all appear to have two beneficial effects, both of which influence the extent to which the systemic inflammatory response is activated. First, they appear to reduce mitochondrial damage, which is perhaps the mechanism for improved recovery of tissue ATP levels. Reduced mitochondrial damage has been shown by decreased levels of cytoplasmic cytochrome c and by decreased activity of caspases, notably caspase 3 and 7, and by decreased apoptosis. Second, they appear to suppress the cytokine response, at least in part by inhibiting the activation of the transcription factor NF- κ B. The activation of NF- κ B is dependent among other factors on the proteasome, a large cellular proteolytic complex that has a regulatory role in the inflammatory process. In parallel investigations, agents that inhibit proteasome activity have also been shown to suppress cytokine production in response to inflammatory stimuli (11-19). Previous research has established many similarities between the inflammatory response induced by sepsis and that induced by hemorrhagic shock. It is becoming increasingly apparent that these similarities may reflect similar changes in the proteasome, a line of research which has been pursued by our laboratories, and which touches on the present investigation in significant aspects.

Glutamine-alanine dipeptide (Dipeptivin, Fresnius-Kabi) has been approved for use and is widely used in Europe. It is cleaved by plasma proteases to glutamine and alanine, and is thus best regarded as a means of administering glutamine. But pharmacologically, it is much more soluble than glutamine, and thus more easily given in intravenous form. It is for that reason a more suitable agent for use during resuscitation. The greater solubility allows administration of a larger glutamine-equivalent dose than glutamine alone. It can be given in a much smaller volume of fluid. Previous studies have been carried out both with this agent and with glutamine in animals. (22,23)

Unfortunately, several large studies carried out in this country and internationally have failed to show that glutamine is effective. A large multicenter study (REDOXS) of the dipeptide and antioxidants in critically ill ventilated patients with multiple organ dysfunction showed no beneficial effect (24). Other studies which have been presented but not yet published have shown similar negative results. Studies in burned patients have, on the other hand, shown generally positive results in reducing infection (25). A study by Grau, et al, showed that the dipeptide when used in critically ill patients on parenteral nutrition lowered infections and improved glycemic control. A critical difficulty with such studies is that glutamine, as a common dietary amino acid, is incorporated into enteral nutrition solutions. Because it is not stable in long-term aqueous solution, it is not used in total parenteral nutrition solutions. Hence, these are in effect glutamine-deficient diets. In short, the question of effectiveness of glutamine in injured patients has not been effectively addressed.

The relevance of this discussion to the present study is that the only form of glutamine approved for US use is the intravenous 3% solution. This requires a liter of fluid to administer 30 grams, which is not an adequate dose for an average adult. It is impractical for field use, although it could be incorporated into hospital practice. The alanine-glutamine dipeptide would be a considerably more useful form of therapy, as it can be given in a much more concentrated form. But from informal communications with representatives of Fresenius-Kabi, which makes the dipeptide for human administration, it is unlikely that the agent will be approved for US use in the near future.

KEY RESEARCH ACCOMPLISHMENTS

3.1. Research Objective #1: Evaluate effectiveness of modified resuscitation

procedures in an experimental model. *Deliverable:* Building on previous work, we have studied glutamine, arginine, and allopurinol under conditions of both sublethal and lethal hemorrhagic shock. We expected to determine the conditions under which these can be used with standard fluid resuscitation regimens and with low-volume resuscitation. The desired outcome of this work was to define a therapeutic strategic in which one or more pharmacologic agents can be given in association with the low-volume resuscitation strategy routinely used today by the military medical services in combat zones. The plan for this objective was to study glutamine during the first year, define the optimum dosage, and then study arginine and allopurinol in the experimental animal model while the clinical studies proceed with glutamine.

1. A number of preliminary studies were carried out under a previous contract with the Department of Defense (W81XWH-06-1-0530). Collaboration continues with the laboratory of Dr. Peter Smith, Kansas University Medical Center, Kansas City, KA, for micro-array analysis of both animal and human genomes.
2. L-arginine appears to play a critical role in the development/regulation of the host inflammatory response to stress or trauma. This amino acid functions as a nitrogen donor in a number of biosynthetic reactions. It also serves as the substrate of inducible nitric oxide synthase, which is intimately involved in the development of an inflammatory response and is upregulated in levels of expression in response to a number of stressful stimuli. There is strong evidence that the decreased availability of L-arginine correlates with reduced levels of expression of the inducible nitric oxide synthase (iNOS) enzyme, probably through reduced levels of translation of mRNA for iNOS. L-Arginine appears to be involved directly in regulation of iNOS activity, and appears to have a significantly greater role than serving simply a substrate for the production of NO.
3. Studies by others on the potential therapeutic use of L-arginine collectively allow the conclusion that L-arginine supplementation can be beneficial in ameliorating the tissue damage produced following experimental hemorrhagic shock. In the investigation outlined here, we have critically evaluated the contribution of L-arginine as a potentially beneficial component of the resuscitation fluid in an experimental rat model of hemorrhagic shock. Our studies were designed to explore the overall patterns of gene expression observed in peripheral blood mononuclear cells following shock with and without treatment with L-arginine administered during resuscitation.
 - a. Male Sprague-Dawley rats, 280-300 gm were purchased from Charles River, Inc (Boston, MA.) Animals were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL), using a standard vaporizer (SurgiVet, Inc. Waukesha, WI). Oxygen was administered at 3L/min. Using aseptic technique, the femoral artery and vein were cannulated using Polyethylene catheters (PE50; Clay Adams, Becton Dickinson and Company, Sparks, MD). Blood pressure and heart rate were monitored continuously by connecting the femoral artery catheter to a pressure transducer and computerized system (Digi-Med, Micro Med Inc,

Louisville, Kentucky), with continuous monitoring. An initial blood sample was taken for analysis, and then shock was produced by withdrawing blood from the femoral artery until the mean arterial pressure (MAP) was 25 mm Hg. Blood pressure was maintained at 25-30 mm Hg for 30 minutes. Depending on the experimental group (see below), resuscitation was carried out by infusing 21 ml/kg Ringer's lactate (LR) over 30 minutes, either with or without supplemental L-arginine, 300 mg/kg. Racemic LR solution was used (Hospira, Inc, Lake Forest, IL). L-arginine was added to the LR in powdered form (Acros Organics, New Jersey), using filtration to sterilize. Following this, half of each group of animals were kept anesthetized for 30 minutes, and then sacrificed by aortic transection after obtaining a blood sample. The other half of each group of animals had the catheters removed and incisions closed, and then were allowed to waken. At four hours post shock, they were re-anesthetized using Isoflurane, a blood sample was drawn, and they were sacrificed by aortic transection.

- b. Analysis of Gene Expression: The rats were randomized into four groups; Sham Group, Shock Group, Shock and lactated Ringer's (LR) Resuscitation Group, and Shock and lactated Ringer's with L-Arginine Resuscitation Group as described above. Three to five animals were successfully analyzed in each group.
- c. The microarray analysis used 31,099 probes. Genomic response was quite marked in all four groups. Even the sham surgical procedure was a strong stimulant to the gene response in white blood cells. This data supports previous work by many others indicating that so-called hemorrhagic shock procedures are, in fact, a combination of trauma and shock.

Table 1: Genomic response		
Experimental group	Genes upregulated	Genes downregulated
Sham at 30 minutes vs sham baseline	4993	7178
Shock at 30 minutes vs shock baseline	4758	7579
Shock + LR at 30 minutes vs shock + LR baseline	5554	7510
Shock + LR + arginine at 30 minutes vs baseline	6143	6325

- d. Microarray analysis was done as follows.

RNA Preparation and Probe synthesis.

Whole anticoagulated blood specimens were collected in EDTA from individual rats following the protocols summarized above. Contaminating red blood cells (which lack significant amounts of RNA) were lysed to facilitate their separation from the peripheral blood mononuclear cells and remove contaminants, including any remaining contaminating heme and protein. RNA extraction was carried out using the Versagene RNA purification kit (Gentra Systems, Minnesota, USA), using the manufacturer's recommended protocol.

The peripheral blood mononuclear cells were added to a detergent/salt solution to lyse and homogenize the cells and at the same time eliminate potential endogenous RNase activity. Homogenization disrupts the cell membranes releasing RNA into the lysis solution, and shears the genomic DNA, decreasing the viscosity of the lysate. Whole blood lysates from blood volumes greater than 3.0 ml were then passed through a Gentra PRECLEAR column to remove the remaining debris after homogenization. The lysates were applied to the Gentra purification column, which binds RNA. Proteins, DNA and other contaminants were washed off. Residual DNA was removed by subsequent on-column DNase treatment, followed by washing to remove DNA fragments and DNAase . Finally, the purified RNA was eluted with DEPC-treated water. The extracted RNA was stored at -80 degrees until used for experiments.

Array Hybridization

Samples for analysis of gene expression levels were processed using the Affymetrix standard Target Labeling Protocol, and were analyzed out at the Microarray facility at University of Kansas Medical Center (KUMC). Prior to experiments, all RNA samples were analyzed for RNA quality and quantity using the Agilent 2100 Bioanalyzer.

The RNA was initially annealed using T7(dT)24 primer, which was then reverse transcribed into cDNA using the Superscript Choice system (Invitrogen). After the first and second strand synthesis, the cDNA extraction was carried out followed by Phase Lock Gel separation (Brinkman Instruments/Eppendorf) and then by DNA precipitation. In- vitro-transcription biotin labeling was achieved using the GeneChip Expression 3'-Amplification IVT Labeling kit. The cRNA generated from either IVT labeling reaction was purified using RNeasy RNA Purification Mini kit (Qiagen) using the RNA Cleanup Protocol according to the manufacturer's instructions. The Biotin-labeled cRNA was quantitated using 260nm/280nm spectrophotometric assays.

The GeneChip was pre-hybridized with 200ul 1x Hybridization buffer at 45C for 10min at 60rpm. Then 200ul denatured Hybridization Cocktail was applied to the GeneChip, which was then hybridized for 16 hr at 45C and 60rpm in the GeneChip Hybridization Oven 640. Hybridized GeneChips undergo low and high stringency washing and R-Phycoerythrin-Streptavidin staining procedures using the GeneChip fluidics station 450. Scanning was conducted using the Agilent Gene Array Scanner. 1x scans were conducted for the GeneChip Expression 3'-Amplification IVT Labeling using a Pixel value = 3um and wave length of 570nm.

e. Data Analysis

The dataset consisted of forty-two high density microarray GeneChips (Rat 230 2.0 which contained 31,099 probes) distributed according the experimental design of this project into three groups (baseline, 30 min, 4 hours) and four experiments (Sham, Shock, Shock and LR, Shock, LR and Arginine). The chips were scanned and analyzed using MAS5 (MicroArray Suite) type of data analysis. Data quality control followed by the statistical

analysis was performed by Affymetrix GeneChip Operating Software (GCOS) and GeneSpring GX 7 (Agilent Technologies), where the initial signals were subsequently normalized per gene and per chip prior to the statistical analysis. The Pearson's correlation coefficients of housekeeping genes were ≥ 0.995 for each comparison in all experiments. The status of specific gene probe (present, marginal and absent) is based on the calculated detection p-value (Student's T test), where p-value under 5% (<0.05) defines the probe as present. In cases where the detection p-value is between 5% and 6.5% (0.05-0.065) the detection interpretation is marginal, lowering the significance level to less than 95%. When the detection p-value exceeds 0.065 (6.5%) the probes were designated as absent and eliminated from the further analysis.

Normalized signals from different groups and experiments were then compared to compute the fold and signal change for each probe. The cut-off fold change of 1.5 and -1.5 was taken for sorted up- and down-regulated genes, respectively. Finally, we combined the up- and downregulated genes from the initial groups to determine the potential genes of the interest. The estimates of the potential peripheral blood mononuclear networks responsive to hemorrhagic shock and resuscitation fluids with or without arginine and corresponding functional analyses were carried out using the Ingenuity Pathways Analysis Program (Ingenuity Systems, www.ingenuity.com).

f. Network Analysis

The data set containing gene identifiers and corresponding Expression Values were uploaded into the Ingenuity Pathways software application. An Expression Value was defined in these analyses as a numerical value that indicates the extent of activity (or relative importance) of each component (specific gene expressed) of the pathway being queried. We used 'ratio' as the expression value type. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. A ratio threshold of 3.0 was established to distinguish genes whose level of expression was significantly differentially regulated relative to controls. These genes, termed focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated, based on the extent of their connectivity.

Canonical pathways are well-characterized metabolic and cell signaling pathways associated with specific clusters of activated genes. Using the Ingenuity Pathways analysis library, the canonical pathways that most significantly correlated with the generated data set were then identified. Genes from the data set that satisfied the threshold ratio cutoff of 3 and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for analysis. The significance of the extent of the association between the actual data set and the specific canonical pathway was evaluated in 2 ways. First, the ratio of the number of genes identified to be significantly regulated and that map to a specific canonical pathway was displayed. Second, Fischer's exact test was used to calculate a p- value determining the probability that the observed association between the

identified genes in the data set and a specific identified canonical pathway could be explained by chance alone.

Table 2: Canonical pathways most differentially affected by arginine

30 minutes	4 hours
cAMP-mediated Signaling	Hepatic fibrosis and hepatic stellate cell activation
G-protein Coupled Receptor Signaling	Wnt/β-catenin Signaling
VDR/RXR Activation	cAMP-mediated Signaling
Hepatic Fibrosis / Hepatic stellate cell Activation	Acute Phase Response Signaling
Glutamate Receptor Signaling	Glycerolipid Metabolism
Wnt/β-catenin Signaling	TGF-β Signaling
LXR/RXR Activation	Nitrogen Metabolism
Cardiac β-adrenergic Signaling	Glutamine Receptor Signaling
Androgen and Estrogen Metabolism	Cardiac β-adrenergic Signaling
Inositol Metabolism	Pentose and Glucuronate Interconversions

Cytokine	Gene	Protein	Control	Arginine	Pathway
IL-1	IL1R1	IL1 receptor	Up-regulated	Down-regulated	APR
IL-1	IL1RAP	IL1 receptor accessory protein	Down-regulated	Up-regulated	APR
IL-1	MYD88	Myeloid depression primary response	Down-regulated	Up-regulated	APR, HC
IL-1, TNF- α	JNK ½	JNK	Down-regulated	Up-regulated	APR
IL-1, TNF- α	MKK 4/7	MKK	Down-regulated	Up-regulated	APR
IL-1, TNF- α	I κ B	I κ B	Up-regulated	Neutral	APR
IL-4	IL-4R	Interleukin 4 receptor	Down-regulated	Up-regulated	
IL-6	SHP2	Tyrosine phosphatase	Up-regulated	Down-regulated	APR
IL-6	JAK2	Janus kinase 2	Dow-regulated	Up-regulated	APR
IL-6	PDK1	Phosphoinositol dependent kinase	Down-regulated	Up-regulated	APR
IL-6	cRAF	Murine leukemia viral homolog oncogene	Down-regulated	Up-regulated	APR
IL-6	ERK ½	MAP kinase	Down-regulated	Neutral	APR
IL-6	Elk1	Member ETS oncogene family	Down-regulated	Up-regulated	APR
TNF- α	TTR	Transthyretin	Up-regulated	Down-regulated	APR
TNF- α	TLR4	Toll-like receptor 4	Down-regulated	Up-regulated	HC
Growth factors	PDGF- α	Platelet-derived growth factor α	Up-regulated	Down-regulated	HF
Growth factors	PDGFR- α	Platelet derived growth factor receptor α	Down-regulated	Up-regulated	HF
Growth factors	PDGFR- β	Platelet-derived growth factor receptor β	Up-regulated	Down-regulated	HF
Growth factors	LEPR	Leptin receptor	Up-regulated	Down-regulated	HF
Growth factors	FGFR1	Fibroblast growth factor receptor 1	Up-regulated	Down-regulated	HF
Growth factors	FGFR2	Fibroblast growth factor receptor 2	Up-regulated	Down-regulated	HF
Growth factors	IFBP5	Insulin-like growth factor binding protein	Up-regulated	Down-regulated	HF
Growth factors	CSF1	Colony stimulating factor 1	Neutral	Down-regulated	HF
NOS	AChR	Cholinergic receptor, muscarinic 1	Up-regulated	Down-regulated	NOS
NOS	PLN	Phospholamban	Up-regulated	Down-regulated	NOS
:NOS	ARG2	Arginase, type 2	Down-regulated	Neutral	LXR
Other	β -catenin	β -Catenin (transcription regulator)	Down-regulated	Up-regulated	WNT
Other	GRM5	Glutamate receptor, metabotropic 5	Up-regulated	Down-regulated	GLUT
	GRIP	Glutamate receptor interacting protein 1	Up-regulated	Down-regulated	GLUT

Table 4: Changes seen in several canonical pathways relating to specific cytokines, comparing resuscitation with L-arginine plus Ringer's lactate, versus Ringer's lactate alone (control).

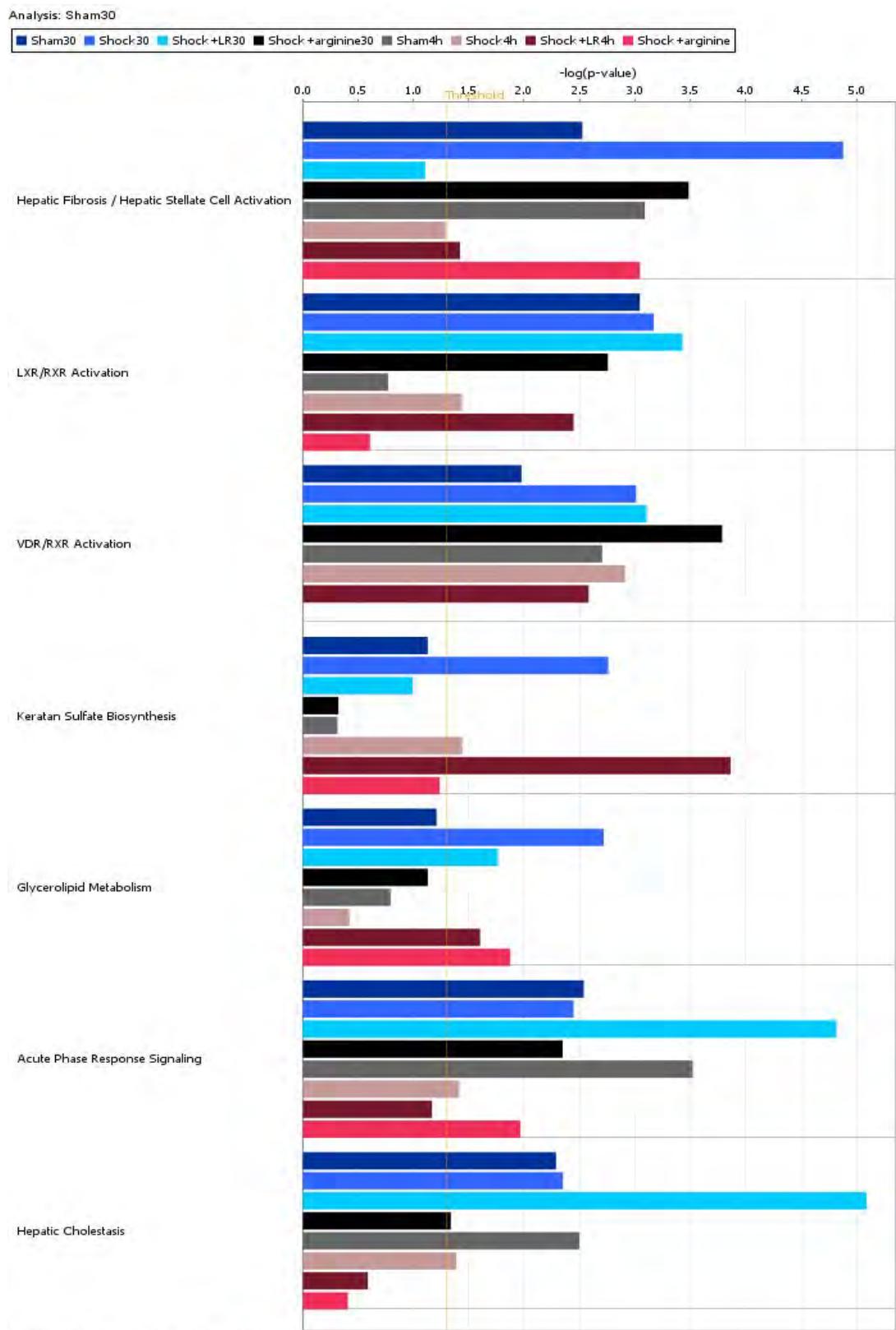
Key to canonical pathways: APR - Acute Phase Response Pathway; HC – Hepatic Cholestasis; HF – Hepatic Fibrosis and Stellate Cell Activation

4. The genome data from the shock experiment described in Objective #1 has been analyzed further, specifically in the analysis of the two shock groups treated with and without arginine. The objective was to determine the differential effect of adding arginine. Also shown here is the relative activation of pathways as measured by up-regulation of genes in those pathways.

As shown in the table below, relative activation of genes is maximal at 30 minutes, and is somewhat attenuated at four hours, except for the ferritin sulfate biosynthesis pathway and the VDR/RXR activation pathway. This is of particular interest in the light of some apparently unrelated work carried out in our laboratories. We surveyed 204 patients admitted to Truman Medical Center, and found that 198, or 97%, were Vitamin D deficient. Studies to determine if this deficiency is present in those patients admitted for trauma are ongoing. This raises the interesting possibility that vitamin D deficiency, particularly common in the inner city population served by TMC, may have an adverse effect on the response to shock and injury.

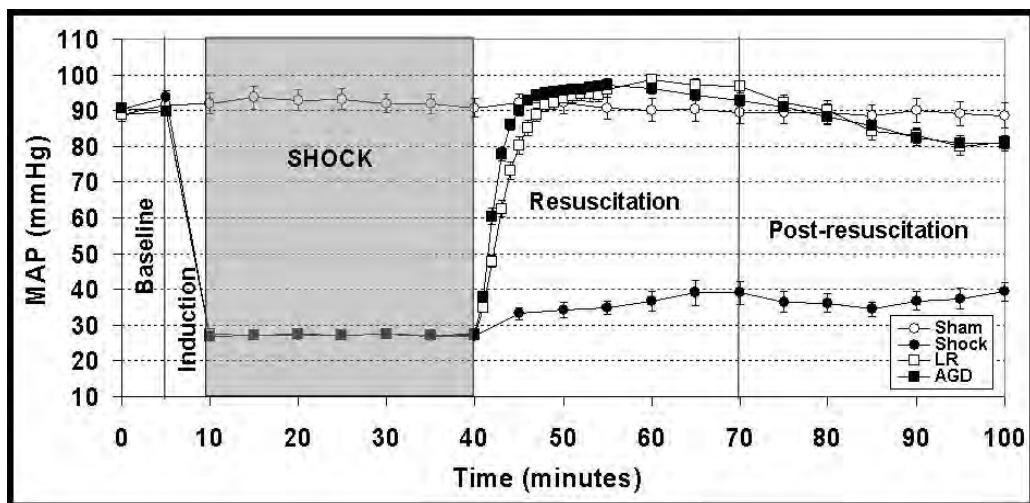
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Table 5. Effect of arginine on gene activation in seven canonical pathways. Relative activation of genes is shown in the seven pathways most affected by shock. Within each group, activation is compared with the baseline values for that group. For each pathway, the first four bar graphs represent sham 30 minutes, shock 30 minutes, shock resuscitated with LR at 30 minutes, and shock resuscitated with LR and arginine at 30 minutes. The second four bar graphs represent the same experimental groups at 4 hours. Most of the pathways, with some exceptions, show an attenuated response at four hours.



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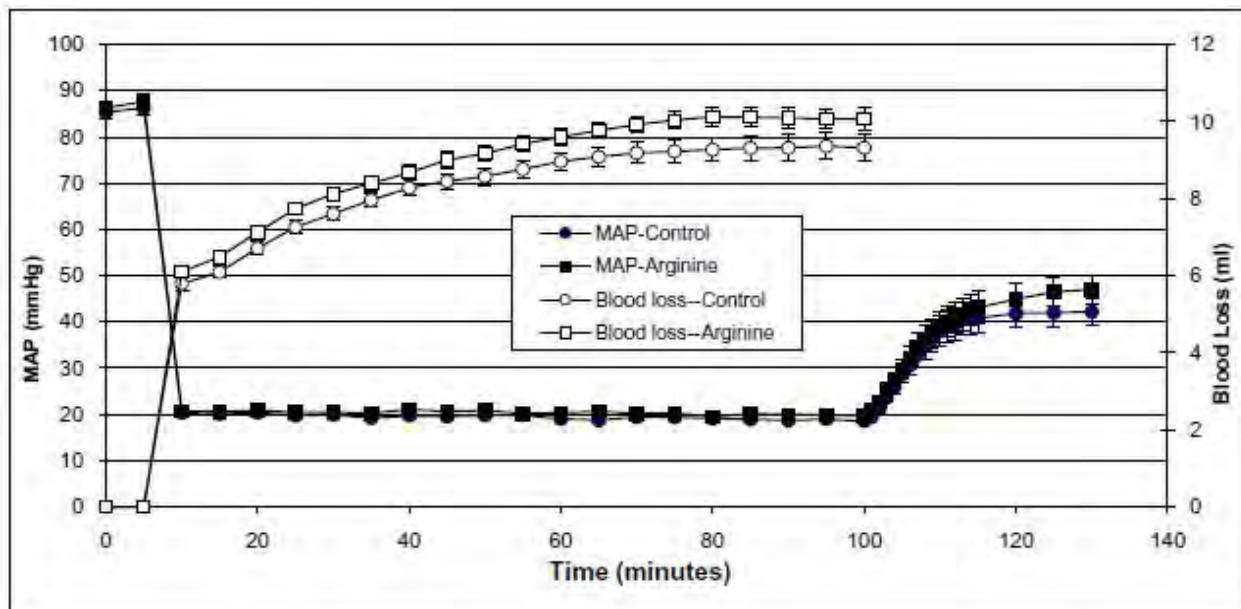
5. It may be well to summarize the applicability of this work to ongoing work under the present study. It is clear that whole-genome studies generate an immense amount of data. While we have evaluated this data with "sentinel gene" analysis and pathway analysis, we have found that these represent a very indirect approach to the question of just how a given agent is affecting recovery from the shock insult. Initial studies in patients, in whom the conditions are not nearly as well controlled as in the experimental model, are even less directly applicable. Going forward, we intend to make use of the RT-PCR technique to look at particular genes that are activated in response to particular agents. For example, glutamine is known to up-regulate the heat shock proteins HSP-25, HSP-70, and HSP-90. Direct measurement of the mRNA for these proteins is proving to be a better method to evaluate the effect of glutamine than studies of a broader array of genes.
6. The figure below illustrates the model developed for use in these experiments. The particular series of experiments illustrated used the dipeptide alanine-glutamine



dipeptide, which is a glutamine equivalent used clinically in Europe and elsewhere (Dipeptivin). Sprague-Dawley male rats, 300-350 grams, were anesthetized with Isoflurane. Cannulation of the femoral artery and vein were carried out. (20) Hemorrhagic shock was induced by withdrawing blood (0.5 ml/min) to lower the blood pressure to 25-30 mm, and maintaining it at that level for 30 minutes. Resuscitation was by lactated Ringer's solution, 21 ml/kg over 30 minutes. Animals are sacrificed 1 hour after the end of the shock and resuscitation period. At that time, tissue samples are obtained and blood removed for analysis.

7. Survival studies were carried out in a similar model, using a much longer shock period to render the preparation lethal in over 50% of control animals. The model is shown in the figure below.
- Male Sprague-Dawley rats, 280-300 gm, were purchased from Charles River, Inc (Boston, MA.) Animals were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL), using a standard vaporizer (SurgiVet, Inc. Waukesha, WI). Oxygen was administered at 3L/min. Using aseptic technique, the femoral artery and vein were cannulated using Polyethylene catheters (PE50; Clay Adams, Becton Dickinson and Company, Sparks, MD). Blood pressure and heart rate were monitored

continuously by connecting the femoral artery catheter to a pressure transducer and computerized system (Digi-Med, Micro Med Inc, Louisville, Kentucky), with continuous monitoring. Shock was produced by withdrawing blood from the femoral



artery until the mean arterial pressure (MAP) was 20 mm Hg. Blood pressure was maintained at 20 mm Hg for 90 minutes. Resuscitation was carried out by infusing 21 ml/kg Ringer's lactate (RL) over 30 minutes, either with or without supplemental L-arginine, 300 mg/kg. There were 20 animals in each of the two experimental groups. Racemic RL solution was used (Hospira, Inc, Lake Forest, IL). L-arginine was added to the RL in powdered form (Acros Organics, New Jersey), using Millipore filtration to sterilize the fluid. At the conclusion of the resuscitation phase, the catheters were removed, the incisions closed, and the animals were allowed to awaken from anesthesia. They were then observed for the next 72 hours. At that time, the surviving animals were sacrificed by aortic transection under anesthesia. The primary end-point of the experiment was non-survival. Secondary end points measured were the amount of blood required to maintain the blood pressure at 20 mm Hg over 90 minutes, and the level of the blood pressure during the resuscitation period.

Statistical analysis: Survival at 24 and 72 hours in the two groups was compared with Chi-squared analysis. Blood pressures and volumes withdrawn were compared using student's t-test.

Results showed that administration of L-arginine in the resuscitation fluid (Ringer's Lactate) promoted overall survival. Survival at 24 hours was observed to be 80% in

the group of rats administered L-arginine as compared with 45% in the control group of rats ($p<0.05$). Survival at 72 hours was 60% versus 30% ($p=0.06$, Chi square analysis). It should be noted that the statistical power of the experiment at 72 hours was much less than at 24 hours, because of the number of deaths in the control group at 24 hours. There were no differences between the groups in weight and cannulation time. The amount of blood required to maintain shock was somewhat greater in the arginine group, but this did not achieve statistical significance. (Table I) Similarly, the rats in the L-arginine group showed a small, increase in the return of blood pressure at the end of resuscitation, again not reaching statistical significance (Figure 1). We conclude from this experiment that the inclusion of L-arginine in the resuscitation fluid has a statistically significant early beneficial and protective effect against mortality following hemorrhagic shock.

Table 5: Results of survival studies with arginine

	Control	Arginine
Rat weight (gram)	286.70±18.02	294.40±21.50
Cannulating Time (minutes)	6.35±0.58	6.80±0.76
Bleeding (ml)	9.32±1.39	10.06±1.56
Survival < 24 hours	9/20	16/20*
Survival > 72 hours	6/20	12/20
<p>Shock: MAP = 20±4 mmHg, 90 minutes Resuscitated over 30 minutes with Ringer's lactate 21ml/kg) with and without L-arginine (300mg/kg) intravenously Data expressed as mean ± SD. *$P< 0.05$ (control vs Arginine).</p>		

8. Cell culture model of low-perfusion state

- a. Ongoing studies on a cell-culture based model, using a standard macrophage cell line (RAW 264.7). This model is based on exposing cultured macrophages to ischemia in a sealed chamber. These cells tolerate reduced oxygen levels to below 10% quite well. But reducing the level of oxygen to 1.5% from the usual 21% produces a significant hypoxic insult. This corresponds to a partial pressure of oxygen of 10 mm Hg. Interstitial fluid is known to have a relatively low oxygen tension; cells normally exist in an environment of as low as 20 mmHg. Measuring the effect of therapeutic agents on hypoxia-challenged cell lines, will provide further evaluation of their possible therapeutic effectiveness.

b. Materials and Methods: RAW 264.7 cells were cultured in DMEM medium and exposed to an atmosphere of 1.5% oxygen and 5% CO₂ for periods up to 48 hours. The cells were grown in Billups-Rothenberg chambers. Control cells were kept in 21% oxygen and 5% CO₂, using a standard incubator.

Cell growth: Cell counts and growth of cells were measured by two different ways, automated cell count after trypsin digestion and total nucleic acid analysis.

Proteasomal enzymatic activity: The three types of proteasomal activity (chymotrypsin-like, trypsin-like, and post-glutamate), were measured using a commercial assay system (Proteasome-Glo, Promega, Madison, WI), using a plate luminometer (Promega). Proteasome-Glo reagent (Promega) of the appropriate type (SucLLVY-Glo for chymotrypsin-like activity; Z-LRR-Glo for Trypsin-like activity; ZnLPnLd-Glo for post-glutamate activity. Plates were incubated for 30 minutes at 370 C. Relative Luminescence Units (RLU) were measured with the plate luminometer.

ATP measurements: In order to determine ATP content of the hypoxic cells, ATP was measured using a modified chemiluminescence assay (Cell Titer-Glo Luminescent Cell Viability Assay System, ProMega Corporation, Madison, WI). After removing the medium from the cell culture wells, the cells were lysed using the Cell Lysis Buffer, then centrifuged to remove debris. The cell lysate was placed in a well, and then the Luciferase Assay Reagent added. The resulting luminescence was read using the luminometer according the manufacturer (Promega). Because this assay system is intended to be used to measure cell viability, rather than ATP levels, it was modified by constructing a standard curve using ATP (Fisher), which was linear over the range to be assayed. The amount of ATP was then determined from the relative luminescence value and the standard curve. Finally, cellular ATP content was calculated using the ATP content of the sample and cell counts as determined above.

Cytokine measurements

To assess inflammatory mediators, studies were done for TNF α , IL1 β , and iNOS, assessing both protein (ELISA) and mRNA (reverse transcription and blotting) and protein levels (Western blot). Levels of TNF α and IL1 β were determined in cell culture supernatant using the Quantikine MELISA kit (R&D System, Minneapolis, MN). NO levels in the supernatant were measured using nitrite levels as an index of nitric oxide synthase (NOS) activity. These were done using the Griese reagent.

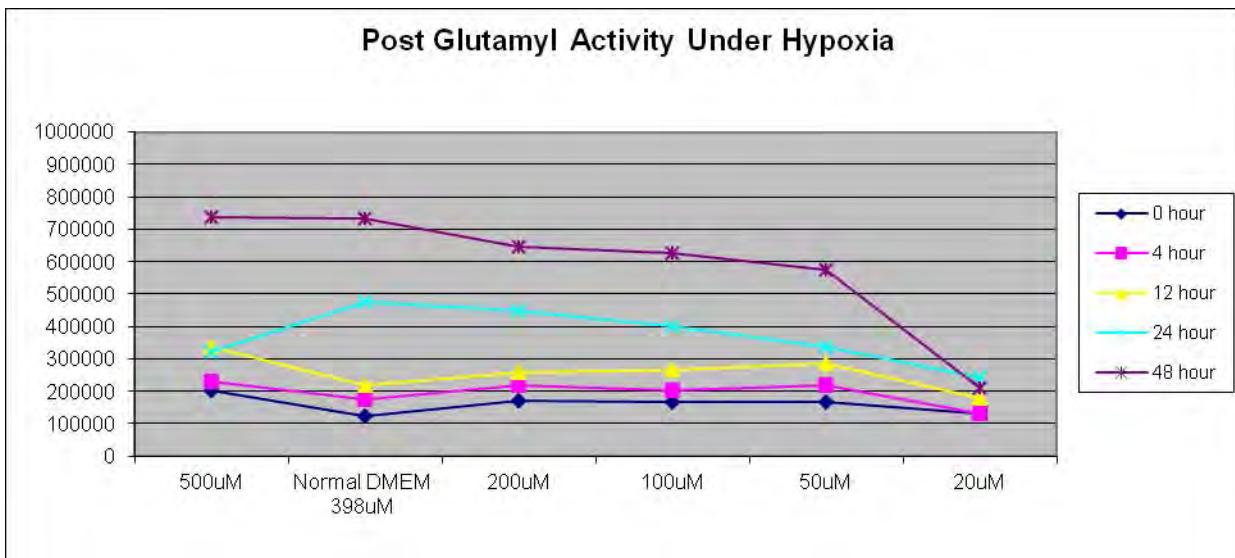
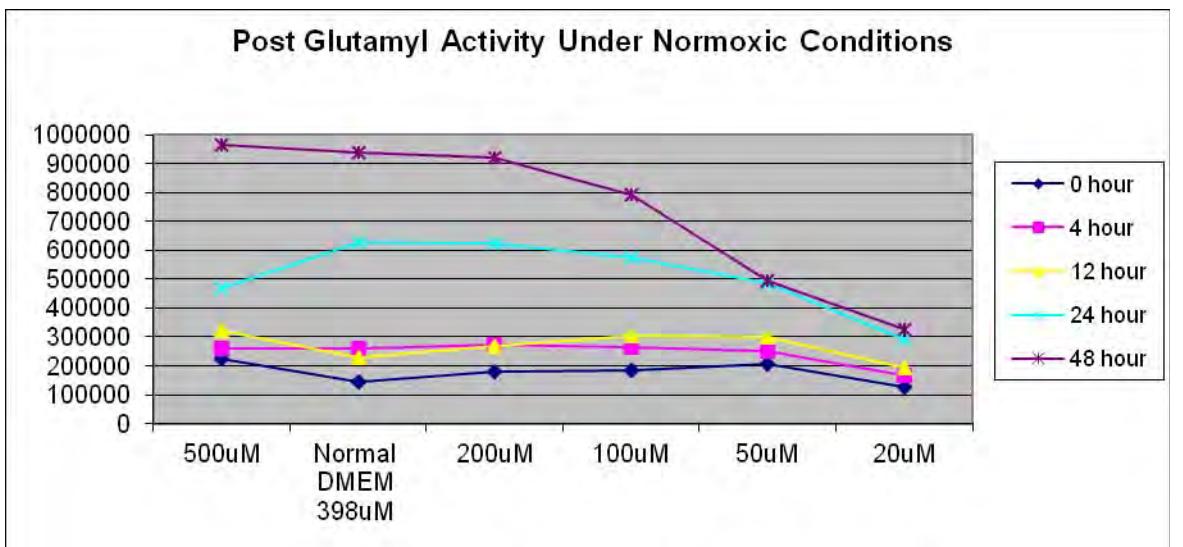
Effects of arginine: The effect of arginine was determined by using arginine-free DMEM medium, to which was added differing levels of arginine (10, 20, 50, 100, 200, 400, and 500 micromolar. The "usual" level of arginine in DMEM medium is 398 micromolar. Cell growth was determined as an indication of cell tolerance of the level of arginine. The three types of proteasomal activity were measured at each level of arginine, after 24 hours of growth. Cells grown in hypoxia were compared with cells grown in normal

oxygen. As controls, cells were grown with D-arginine both in hypoxia and normoxia.

c. Results: . Cells tolerated exposure to hypoxia at 1.5%. Growth was about 15% less than normal controls, measured either by cell count or nucleic acid analysis. Their growth slows markedly thereafter as compared with normoxic controls. But adenosine triphosphate (ATP) levels within cells left chronically ischemic for up to 24 hours had similar ATP content per cell as cells grown in normoxia. This result is consistent with the hypothesis that cells retain their ATP content under hypoxia, and that they may grow only as fast as they are able to keep their ATP content constant. Considering this finding and previous research, we intend to use ATP as an indicator of the degree to which cells have recovered from the hypoxic insult. Going forward, our experiments will incorporate measurement of ATP levels in target organs, particularly the liver.

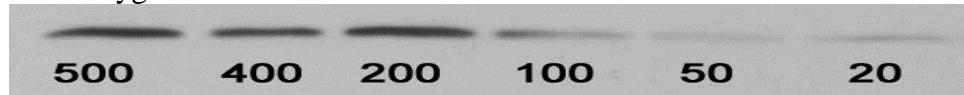
d. Proteasomal activities in the hypoxic cells showed, as compared with controls, a twofold increase in chymotrypsin-like activity, unchanged trypsin-like activity, and somewhat post-glutamate activity was decreased modestly, about 15%. These changes were accompanied by up-regulation of mRNA for the subunits LMP-7 and LMP-2, and appearance of the corresponding proteins. These changes are similar to those produced by LPS stimulation. Cytokine secretion and mRNA for cytokines were less evident. TNF α , IL-1 β , and IL-6 were not stimulated by hypoxia, while iNOS mRNA and protein level were increased significantly. Nitrite production remained at low levels during hypoxia.

e. Proteasomal activities were strongly influenced by the arginine concentration in the growth medium. As can be seen in the figures below, the post-glutamyl activity of the proteasome was strongly influenced by arginine concentration both in the normoxic and hypoxic state. Similar findings were noted for the chymoglycyl-like activity and the trypsin-like activity of the proteasome.

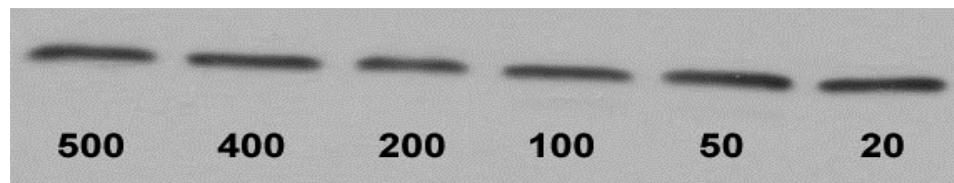


f. Studies strongly suggest that stress-induced modifications of host responses are associated with a change in the proteolytic subunits of the multifunctional cellular proteasome. This was confirmed in the hypoxic macrophage model, in which the chymotrypsin-like activity, was far more increased in hypoxia than were the trypsin-like and post-glutamate activities. As noted above, this change in activity was found to be responsive to changes in arginine concentrations. This change appears to be caused by changes in the composition of the proteasome itself. As an example, levels of LMP2, a proteasomal proteolytic subunit that is induced under stress, was found to be highly responsive to the concentration of arginine. In the Western blot below, bands reflect relative levels of LMP2 protease and the numbers indicate the

arginine concentration in culture media. This data is from cells subjected to 1.5% oxygen for 24 hours.

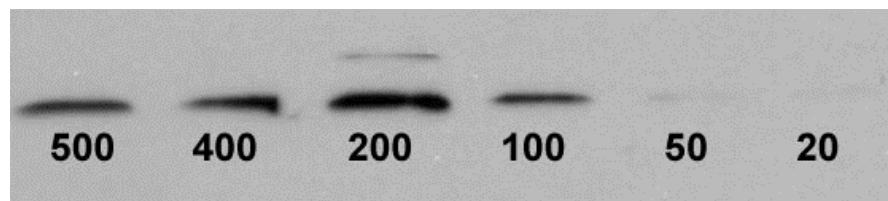


The following Western blot is from cells treated with differing concentrations of arginine, but grown under normoxia (21% oxygen) for 24 hours:

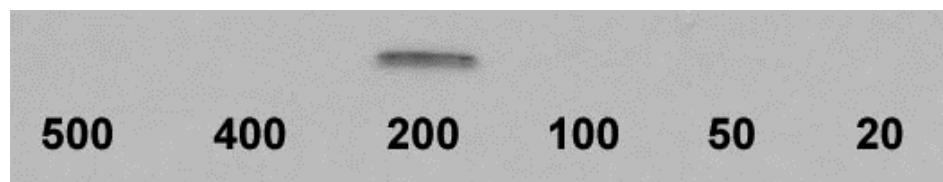


The difference between these two is striking. Under normoxia, LMP2 concentration is unaffected by arginine concentration; under hypoxia, LMP2 concentration is strongly dependent on arginine concentration.

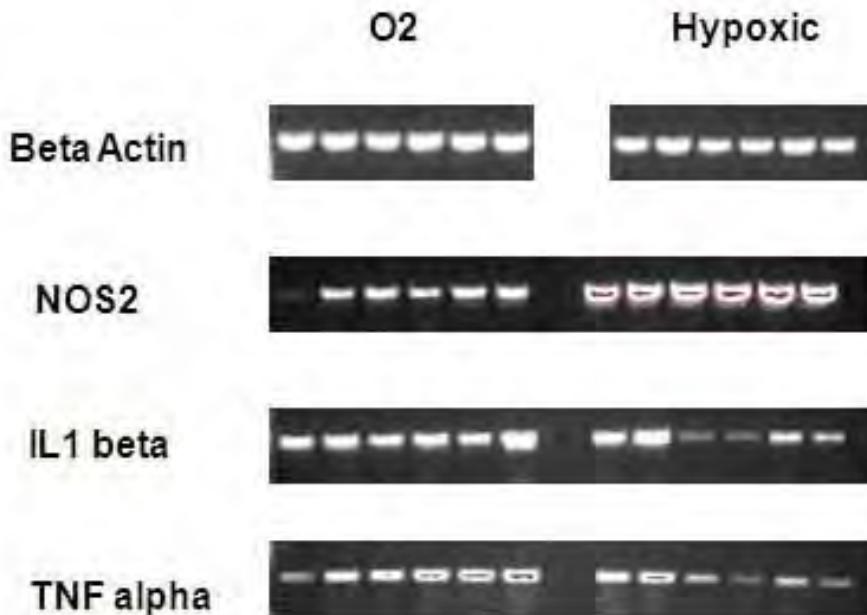
Another of the proteasome subunits is LMP7. This is measured in the next Western blot, from cells grown under 1.5% oxygen for 24 hours. Here, again, the concentration of LMP7 is dependent on arginine concentration, and there appears to be a maximum activity corresponding to an arginine concentration of 200 micromolar.



Finally, the Western blot below is from cells grown under normoxia, and LMP7 is measured. This subunit is barely expressed at all under normoxia, and is seen only at the optimum arginine concentration of 200 micromolar.



g. Cytokine levels also reflect this dependence on arginine. The following is from reverse transcription PCR (RT-PCR) studies of cytokines; beta-actin is used as a reference. The levels of arginine in the bands are, from left to right, 500, 400, 200, 100, 50, 20 micromolar arginine in the growth medium. As



expected, mRNA for NOS2 was upregulated in hypoxia. Somewhat surprisingly, it appears to be inversely dependent on arginine concentration under normoxia. In this set of studies, mRNA for the two classic cytokines, IL-1 beta and TNF alpha, both appear to be inversely proportional to arginine concentration under normoxia, and directly proportional to arginine concentration under hypoxia. These results correlate strongly with the results seen in the studies of proteasomal subunit concentrations.

8. To summarize the place of the cell culture model in this research, we anticipate that it will allow isolation and study of mechanisms of cellular response to hypoxia, and hence to low flow, in a model far more simplified than the whole animal mode. It does not replace the whole-animal model by any means, since much is going on in shock that cannot be reproduced in the more simplified model. But as a tool to allow us to look into the cellular response mechanisms, we feel that it will continue to be productive and to provide new insights into the effect of potential therapeutic agents. One of the .

3.2. Research objective #2: Evaluate efficacy of a modified resuscitation procedure in patients following shock. *Deliverable:* We expected to define the effectiveness of the experimental therapeutic agent glutamine, used in conjunction with the Advanced Trauma Life Support resuscitation protocol, in reversing shock-dependent post-resuscitation changes in patients. The effect of glutamine was to be assessed by measuring changes in levels of expression of signature genes, cellular responses, cellular damage and clinical profiles. The agent was administered at the time the patient was

admitted to the trauma center, and efficacy determined relative to placebo-treated controls. Studies were carried out with glutamine in patients with mild to moderate trauma, under a full consent protocol. Unfortunately, such patients, while suitable for preliminary studies, do not exhibit the clinical signs and symptoms of hemorrhagic shock, nor do they show the inflammatory sequelae. Pursuing these studies under a waiver of consent protocol met opposition from the Institutional Review Board (IRB) of the University of Missouri – Kansas City. The resulting protocol was reviewed and approved by the Human Research Protection Office (HRPO), Office of Research Protections (ORP), U.S. Army Medical Research and Materiel Command (USAMRMC). But the studies were ultimately unsuccessful, because it proved extremely difficult to enroll patients into the study. As noted in the initial discussion, during the time period covered by these studies, other studies were published which failed to show efficacy of glutamine administered as the dipeptide alanine-glutamine to critically ill patients. These studies were not directed towards injury or hemorrhagic shock, and it appears that the question of glutamine efficacy in injured patients remains unanswered.

Several conclusions can be drawn from these limited pilot studies. First, the genomic response is maximal at the first hour following resuscitation, with progressively less response at 24 hours and thereafter. Second, there is broad response across the genome, but large responses (four-fold up or down) are confined to a relatively smaller group of genes. Third, many of the genes involved in inflammation are highly up-regulated following trauma.

Working from the results of animal studies, the genes of interest will be different for the three different agents. During the initial studies of glutamine, heat shock protein response (Hsp25, Hsp70, Hsp90) has been advocated as a suitable agent for assessing response. iNOS has been noted in preliminary animal studies to be strongly downregulated following glutamine treatment, an unexpected finding which has not been confirmed in patient studies.

It was the intent of these studies to administer glutamine during or shortly after resuscitation to patients being admitted for injuries. However, the study has required informed consent, and the patients who were studied all had lesser degrees of injury. Patients in clinical shock are not generally able to give informed consent. The present studies have not, therefore, resolved the question of whether or not glutamine will be effective as an adjunct to resuscitation from hemorrhagic shock.

REPORTABLE OUTCOMES

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS AND PRESENTATIONS (Italicized items in previous reports)

1. *Yang R, Tan X, Thomas AM, Shen J, Qureshi N, Morrison DC, Van Way CW 3rd. Crocetin inhibits mRNA expression for tumor necrosis factor-alpha, interleukin-1 beta, and inducible nitric oxide synthase in hemorrhagic shock. JPEN J Parenter Enteral Nutr.* 2006 Jul-Aug;30(4):297-301.
2. *Yang R, Tibbs BM, Chang B, Nguyen C, Woodall C, Steppacher R, Helling T, Van Way CW 3rd. Effect of DHEA on the hemodynamic response to resuscitation in a porcine model of hemorrhagic shock. J Trauma.* 2006 Dec;61(6):1343-9.
3. *Yang R, Van Way CW III, Tan X, Thomas A, Shen J, Steppacher R, Qureshi N, Morrison DC. Alanine-glutamine dipeptide inhibits expression of inflammation-related genes in hemorrhagic shock. Clinical Nutrition Week of the American Society for Parenteral and Enteral Nutrition, Dallas. February 12-15, 2006. (Oral presentation). Clinical Nutrition week., 2006, p145. (Abstract of Distinction)*
4. *Thomas A, Yang R, Van Way CW III, Quresh N. A Preliminary micro-array study of the response to hemorrhagic shock and to therapy with crocetin and alanine-glutamine dipeptide. Kansas City Area Life Sciences. March 30, 2006. Kansas City Life Sciences. 2006, p163. (Abstract)*
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9. *Pontes-Arruda A, Van Way CW III. Feeding During Sepsis: What Works, What Doesn't. Clinical Nutrition Week of American Society of Parenteral and Enteral Nutrition, Jan 27-31, 2007 (Phoenix, Arizona). (Presentation and abstract)*

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14. Rockwell CE, Morrison DC, and Qureshi N. *Lipid A mediated Tolerance and Cancer therapy*. In *Tolerance in Cancer Therapy*. (Invited Chapter, in press) 2008.
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17. Van Way, CW Yang R, Vernon K, Qureshi N, Morrison D, Smith P. *Gene Activation In White Blood Cells Following Arginine Administration In A Model Of Hemorrhagic Shock*. American Society for Parenteral and Enteral Nutrition, Nutrition Week, Feb 1-4, 2009. (New Orleans, LA) (Presented by C. Van Way)
18. Yang, R, Tan X, Kenney RJ Jr, Thomas AM, Landis M, Qureshi N, Morrison DC, Van Way CW III. *Hemorrhagic Shock in the Rat: Comparison of Carotid and Femoral Cannulation*. Journal of Surgical Research, 144:124-126, 2008.
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20. Van Way, CW Vernon K, Qureshi N, Morrison D. *Influence of Arginine upon Hypoxia-Induced Changes in Proteasomal Structure and Function in RAW 264.7*

Cells. American Society for Parenteral and Enteral Nutrition, Nutrition Week, Feb 8-12, 2010. (Las Vegas, NV) (Presented by C. Van Way)

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25. Reis J, Guan XQ, Kissellev AF, Papasian CJ, Qureshi AA, Morrison DC, Van Way CW III, Vogel SN, Qureshi N. LPS-Induced Formation of Immunoproteasomes: TNF- α and Nitric Oxide Production are Regulated by Altered Composition of Proteasome-Active Sites. *Cell Biochem Biophys* 60:77-88, 2011.
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27. Yang, RL, Vernon K, Joseph S, Qureshi N, Morrison DM, Smith, P, Van Way, CW 3rd. Pathway Analysis of Gene Activation in White Blood Cells Following Arginine Administration in A Model of Hemorrhagic Shock. In revision, JPEN
28. Van Way CW 3rd, Vernon K, Qureshi N, Morrison DM. Hypoxia Induces Release of Inflammatory Mediators Together with Changes in Proteasomal Structure and Function in RAW 264.7 Cells. In preparation.

CONCLUSION

This study has rested on the basic hypothesis that agents given during and shortly after resuscitation can favorably influence the inflammatory response following hemorrhagic shock and injury, and favorably influence survival. To date, the hypothesis has been shown to be true in the animal model. In particular, glutamine, alaninine-glutamine dipeptide, and arginine, but not allopurinol, were all found to enhance survival in the animal model.

Work continues to determine optimum doses of therapeutic agents to be used during and after resuscitation. Glutamine, because of solubility considerations, is limited to a relatively fixed dose both in the animal model and in humans. Arginine and allopurinol are more soluble, and can be given in varying doses. Studies are going forward on arginine, to be followed by allopurinol.

Clinical studies are ongoing to validate these hypotheses in patients. Currently, glutamine is being administered to injured patients. Future studies will employ arginine, and then allopurinol.

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Appendix I

Publications, Presentations, and Patents from Shock Trauma Research Center, UMKC, 2010-2013

The research reported in the document is within the Shock Trauma Research Center of the University of Missouri, Kansas City. The following list is of the publications and other activities from the Center over the past year. While many are not relevant to the current contract, a number of them deal with sepsis, shock, and related issues.

Publications in Peer-Reviewed Journals

1. Pollock G, Pesto MM, Sirridge M, Van Way CW III,. Disclosing Medical Errors in Missouri: How to Say “I’m Sorry.” Missouri Medicine, 107:272-278, 2010. (Ranley Award, Best Individual Article, U. of Missouri School of Journalism)
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Presentations (Includes Published Abstracts)

1. Van Way, CW Vernon K, Qureshi N, Morrison D. Influence of Arginine upon Hypoxia-Induced Changes in Proteasomal Structure and Function in RAW 264.7 Cells. American Society for Parenteral and Enteral Nutrition, Nutrition Week, Feb 8-12, 2010. (Las Vegas, NV) (Presented by C. Van Way)
2. Qureshi N, Reis J, Guan X, Qureshi AA, Papasian CJ, Van Way C, Vogel SN Morrison DC. Effective proteasome inhibitors for inflammation and septic shock. Oral Presentation, Ubiquitin Drug Discovery & Diagnostics Conference, 2010 (Philadelphia, PA)
3. Van Way, CW III. The proteasome as a proposed locus for nutrient modulation of gene expression.. European Society for Clinical Metabolism and Nutrition (ESPEN), 5-8 September, 2010 (Nice, France).
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10. Van Way, CW III. A Possible Role for Arginine in the Proteasomal Regulation of Inflammatory Cytokines. PENSA2011 (Parenteral and Enteral Nutrition Society of Asia) October 15, 2011. (Taipei, Republic of China)
11. Van Way, CW, III. The Use of Nutrients in the Resuscitation of the Patient in Hemorrhagic Shock, PENSA2011 (Parenteral and Enteral Nutrition Society of Asia) October 15, 2011. (Taipei, Republic of China)
12. Van Way, CW III. New Requirements for Surgical Residency Training. ACGME National Conference, March, 2012. (Orlando, FL)
13. Van Way, CW III. The Crisis in Graduate Medical Education: Will We Have Enough Surgeons? Missouri Chapter, American College of Surgeons, June 10, 2012 (Lake Ozark, Missouri)

14. Van Way, CW III. From Troy to Kabul. Military Medicine from Antiquity to the 21st Century. Surgical Biology Club III, American College of Surgeons, September 30, 2012. (Chicago, IL)
15. Serrone R, Van Way III C, Geehan D, Loveless B, Frankel E. Persistence of Vitamin D Deficiency in Patients Admitted to a Midwestern Urban Hospital. American Society for Parenteral and Enteral Nutrition, Clinical Nutrition Week, February, 11, 2013. (Phoenix, AZ) Poster presentation.
16. Daley BJ, McClave SA, Cherry-Bukowiec J, Gramlich L, Van Way CW III. Status of Nutrition Training in Graduate Medical Education: Survey of Residency Program Directors. American Society for Parenteral and Enteral Nutrition, Clinical Nutrition Week, February 11, 2013. (Phoenix, AZ) Poster presentation.